Double Patenting Rejection

Claims 1-4 have been rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1 and15 of U.S. Patent No. 6,204,286. Submitted herewith is a Terminal Disclaimer to overcome this rejection, as U.S. Patent No. 6,204,286 and this application are commonly owned.

Applicants believe that, in view of the remarks made above and the Terminal Disclaimer that is submitted herewith, this application is in condition for allowance. Reconsideration and allowance of claims 1-4 is respectfully requested.

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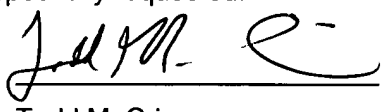
By: 

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LAS, a Novel Selective Estrogen Receptor Modulator with Chemopreventive and Therapeutic Activity in the *N*-Nitroso-*N*-methylurea-induced Rat Mammary Tumor Model[1]

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ABSTRACT

The *N*-nitroso-*N*-methylurea-induced rat mammary tumor model was used to conduct two types of studies: a prevention study designed to test the ability of the novel selective estrogen receptor modulator lasofoxifene (LAS) to inhibit the development of mammary tumors, and a treatment study designed to test the inhibitory effect of LAS on the growth of established tumors. The prevention study indicated that LAS markedly delayed the emergence of *N*-nitroso-*N*-methylurea-induced tumors to an extent similar to that obtained by the established antiestrogen tamoxifen (TAM). At the highest dose administered, both TAM and LAS reduced tumor incidence by 75% and total tumor number by 90% relative to the controls. LAS also reduced the multiplicity of tumors, *i.e.*, the mean number of tumors per rat, and resulted in substantially smaller total tumor burden. In the treatment study, LAS significantly inhibited tumor growth compared with the controls. In addition, whereas none of the untreated tumors regressed completely over the experimental period, 40% of LAS-treated tumors regressed by >50% at the highest dose (10 mg/kg daily). The results of this study in a rat mammary tumor model indicate that LAS has both chemopreventive and chemotherapeutic effects quantitatively comparable with those of TAM.

INTRODUCTION

Antiestrogens, exemplified by TAM[3] (1, 2), are extremely useful agents for the treatment of advanced breast cancer with a response rate of ~34% (1, 2). TAM has also proven very effective in the adjuvant treatment of breast cancer and produces a ~47% reduction of recurrence of estrogen-positive breast tumors upon 5-year treatment (1, 3). Three randomized controlled trials have examined the effectiveness of TAM in the prevention of breast cancer in high-risk patients (4–6). The largest of these trials indicated a significant reduction of risk for breast cancer and this has led to the approval of TAM by the Food and Drug Administration "to reduce the risk of breast cancer in women at high risk of breast cancer" (6). However, the two European studies, with different and smaller target populations, found that TAM did not reduce the risk of breast cancer, thus, different subsets of women may be more or less sensitive to the effects of TAM (4, 5). Some possible reasons for the different conclusions in the three studies have been discussed in recent reviews (7, 8).

Although the antitumor activity of TAM results from its antiestrogenic effects, TAM also acts as an estrogen receptor agonist in other tissues. Thus, although TAM reduced the occurrence of new breast cancers by 45%, it also significantly reduced the frequency of bone fractures in the women entered in the breast cancer prevention trial but increased estrogenic side effects including endometrial cancer and venous thromboembolism (4–6). This finding has led to efforts to identify and develop novel SERMs, synthetic compounds that antagonize the actions of the natural ligand, estrogen, in some tissues, especially breast tumors, but act as an estrogen receptor agonist in others (2, 9). SERMs have potential in the treatment of osteoporosis, the treatment and prevention of breast cancer, and the prevention of heart disease. An ideal antiestrogen would retain the positive effects of estrogens on hot flashes and vaginal dryness as well as long-term benefits on bone and the cardiovascular system, reduce breast cancer incidence, and lack adverse effects such as increased endometrial thickening or vaginal bleeding. Raloxifene, a SERM approved for the treatment of osteoporosis, was shown recently to significantly reduce the risk of estrogen-positive breast cancer and represents significant progress toward this goal (10).

LAS (CP-336,156) is a new p.o. active, nonsteroidal antiestrogen in clinical trials for the treatment of osteoporosis (11). Preclinical studies indicate that it prevents lumbar vertebral bone loss in the OVX rat model, with greatly enhanced potency relative to raloxifene (11, 12). A similar inhibition of bone loss in orchidectomized rats was observed at doses as low as 10 μg/kg a day (13). LAS also lowers serum cholesterol levels without induction of uterine hypertrophy in rat models (11–13). This study examines the effect of LAS on NMU-induced mammary tumors in rats. In this model, female rats receive a single dose of the carcinogen NMU and develop mammary carcinomas several months later (14). The tumors induced by NMU are more likely to be estrogen dependent and are histopathologically more similar to human breast cancers than those induced with dimethylbenzanthracene (15). Both the chemoprotective effect of agents as measured by tumor incidence and the effect on established tumors as measured by inhibition of tumor growth were assessed in this model.

MATERIALS AND METHODS

Reagents. TAM citrate [CAS #54965-24-1] was purchased from Sigma Chemical Co., St. Louis, MO. NMU [CAS #684-93-5] was purchased from Ash Stevens, Detroit, MI. NMU was dissolved in a few drops of 3% acetic acid and diluted with distilled water to give a stock solution of 10 mg of NMU/ml, which was administered within 2 h of preparation (14). LAS, designated previously as CP-336,156, was prepared as described (11).

Animal Care and Adherence to Guidelines. The experimental protocol used (see below) was approved by the American Health Foundation Institutional Animal Care and Use Committee. Animal care was conducted with strict adherence to institutional guidelines and to guidelines specified in the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services publication No. 85-23, 1985). Three rats were housed together in a polyethylene cage that contained wood shavings and was covered with a filter top. The animal room was controlled for temperature (24 ± 2°C), light (12-h cycle), and humidity (50%). Diets were provided in powdered form, and tap water was provided *ad libitum*. Stainless steel pendulum powder feeders (Lab Products, Maywood, NJ) were used to prevent the scattering of food.

Induction of Experimental Mammary Tumors. Virgin female Sprague Dawley rats at a starting age of 35 days (Harlan Sprague Dawley, Indianapolis, IN) were quarantined and maintained on the standard Open Formula Rat and Mouse Ration (NIH-07) diet (4.5% fat, 23.5% protein, 50% carbohydrate, and 4.5% fiber; Zeigler Bros., Gardners, PA) until they were 43 days old, when

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[3] The abbreviations used are: TAM, tamoxifen; SERM, selective estrogen receptor modifier; NMU, *N*-nitroso-*N*-methylurea; AC, adenocarcinoma; GH, growth hormone.

they were placed on the experimental diet [Teklad 4% fat Chow (Teklad 7001); Harlan Teklad, Madison, WI]. This is an open formula grain-based diet routinely used in chemoprevention studies. Details of the diet composition can be obtained from Teklad@Teklad.com. All rats were then assigned to 1 of 16 experimental groups of $n = 20$ rats (prevention study) or $n = 12$ rats (treatment study) by recognized randomization procedures to equalize initial weight. At 55 days of age, all rats received a single dose (47 mg/kg body weight) of NMU by tail vein injection. The subsequent experimental protocol consists of two parts, a prevention protocol and a treatment protocol as described below.

Prevention Protocol. Rats induced to develop mammary tumors with NMU as described above (20/group) were treated with LAS or TAM 2 weeks after induction and dosed every day for a total of 8 consecutive weeks with either vehicle (10% ethanol) or the indicated dose of TAM or LAS. The dosing initially was by s.c. injection but in some animals this caused the formation of a small bleb at the site of injection that slowly resolved. For this reason the dosing was switched to oral (gavage) administration for the second 4 weeks of the dosing period. Oral gavage was by use of a 3.5-inch 18-gauge blunt-tipped feeding tube. Animals were palpated once a week for tumors for a period of 18 weeks, and the position and date of appearance of palpable tumors were recorded. All animals were weighed weekly for the first 10 weeks. Weights were then obtained biweekly for the remaining 8 weeks. In this manner, a cumulative record of body weight change and tumor incidence was generated. The observation period continued after the end of the 8-week treatment schedule until the end of the experiment at 126–133 days after the initiation of dosing. Tumor volumes among the treatment groups were determined by measuring 3 diameters and calculating volume using the following formula: $H \times L \times W \times 0.524$ (approximately an ellipse) in centimeters. The mean and median tumor volume for each treatment group was calculated at termination.

At the conclusion of the experiments a detailed necropsy was performed. Mammary tumors, both palpable and nonpalpable (but grossly visible) were examined grossly and by microscopy. The gross necropsy included an initial physical examination of the external surfaces and all orifices followed by an internal examination of tissues and organs *in situ*. The entire mucosal surfaces of the esophagus, stomach, small and large intestines, and rectum were opened and examined before fixation. All gross lesions were recorded in narrative, descriptive terms, including location, size, number, shape, color, and texture.

During this study, termination rats were killed by carbon dioxide asphyxiation, and mammary tumors (classified as palpable or nonpalpable but grossly visible) were excised and the two largest diameters measured with vernier calipers. The tumors were then fixed in 10% buffered formalin, embedded in paraffin blocks, and stained with H&E for histological examination. Histological diagnosis of mammary tumors was based on the criteria outlined by Young and Hallowes (16) and Russo *et. al.* (17). In this study, 93% of the total number of palpable tumors were adenocarcinomas.

Treatment Protocol. Rats (6 groups of 12 per group) were initiated with NMU as described above and monitored for tumor formation. During the period 8–15 weeks following initiation, at the appearance of the first tumor ≥ 1 cm in diameter, treatment with vehicle (controls), TAM, or LAS at the indicated doses was initiated by daily oral gavage. Tumor diameters were measured in three dimensions at the onset of treatment and every 2 days thereafter with vernier calipers. All rats were sacrificed at 20 weeks or earlier in the case of large necrotic tumors, in accordance with NIH Animal Welfare Regulations.

Statistical Analysis. Latency, defined as the mean time to the first palpable tumor following NMU administration, was assessed by generating tumor-free survival curves for each group (18–20). Rats with no tumors at termination were assigned to latency on day 130. The latency of tumors in each treatment group was then compared with the controls by using the log-rank test.

Tumor incidence (expressed as the percentage of tumor-bearing animals) was compared among the groups by using the χ^2 test. Overall dose-related associations between tumor incidence and treatment dose were tested by using Armitage's test for linear trends in proportions (21, 22). Tumor volume and multiplicity were compared among the groups using one-way ANOVA followed by Dunnett's multiple comparisons test, where each treatment is compared with the control (23).

In the treatment study, tumor volume was measured over time, and each tumor was assigned at termination to one of four categories: progressive (>100% increase), partial regression (>50% decrease), complete regression (tumor disappeared completely), or stable (all others); and the distribution for each group was assessed by using the χ^2 test. In addition, the mean and median percentage change in tumor volume from the baseline was calculated for each group and analyzed by the nonparametric median test and ANOVA followed by Dunnett's test.

The overall weight gains in the animals of all groups were compared by using single-classification ANOVA with repeated measures, followed by Dunnett's test (23–25). The test of interest was the interaction between weight and time to evaluate the null hypothesis of no difference in weight gain over time among the groups. Pairwise comparisons among the groups were also conducted.

All statistical tests were one-tailed and were considered statistically significant at $P < 0.05$. Significance tests for all pairwise comparisons were adjusted for multiple comparisons by multiplying the actual P by the number of comparisons made for the evaluation of statistical significance.

Determination of LAS Concentrations in Plasma. Plasma samples were analyzed using a validated method on a SCIEX API$^{III\text{-}plus}$ LC/MS/MS instrument by Cedra Corp. An aliquot of rat plasma (0.2 ml) with the authentic internal standard (CP-324,098) was precipitated using 0.4 ml of acetonitrile. Following centrifugation, an aliquot (1–10 μl) of supernatant was injected onto a 2.2 × 4.6-mm SCX cartridge with mobile phase consisting of acetonitrile: water:trifluoroacetic acid (4:1:0.005). The column eluent was analyzed using heated nebulizer ion source of the SCIEX API$^{III\text{-}plus}$ at m/z 414 → 97.9 for LAS (retention time, 1.15 min) and m/z 428 → 97.9 for the internal standard (retention time, 1.10 min). The LAS calibration curve consisted of six authentic standards added to the control rat plasma and analyzed in duplicate at concentrations of 1.00, 2.00, 10.00, 50.0, 200, and 400 ng/ml. The acceptance criterion for standards used in the curve was +/−20% of the nominal value (% absolute deviation). As an additional check on accuracy, independent quality control samples were prepared in rat plasma at concentrations of 3.00, 80.0, and 300 ng/ml and analyzed along with the unknowns in every assay. The assay was accepted only if the quality control samples were within +/−20% of the nominal value. The stability of LAS in rat plasma during storage was determined at three different concentrations by examining standard samples stored at the same condition as the test samples. The mean concentration value of the authentic stored standards added to rat plasma did not deviate from the initially determined mean concentration by >15% for at least >3 months.

RESULTS

Chemopreventative Effects. Treatment with LAS or TAM produced a marked delay in NMU-induced tumor appearance (Table 1; Fig. 1, *A* and *B*). For example, by week 12 post-NMU, 42% of the control rats exhibited palpable tumors compared with 10%, 25%, 20%, and 0% tumors for LAS at 0.1, 1.0, 3.0, and 10.0 mg/kg body weight, respectively. The first tumor appeared in the controls at 6 weeks, whereas in the LAS-treated groups the first tumor appearance was at weeks 10, 7, 11, and 17 for groups 0.1, 1, 3, and 10.0 mg LAS/kg, respectively. As reported previously (26), TAM also delayed the emergence of tumors in a dose-dependent fashion (Table 1; Fig. 1*B*).

When adjusted for multiple comparisons, cumulative tumor incidence curves for the two highest doses of TAM and the highest dose of LAS were significantly suppressed compared with the controls (Fig. 1, *A* and *B*). The mean latency for the controls was 101 days compared with 133 and 130 days for high-dose LAS and TAM respectively, indicating that exposure to SERMs for 8 weeks delayed the onset of tumor appearance by ~30 days (Table 1). There were no clear dose-related effects of TAM or LAS on tumor latency.

Tumor incidence (the percentage of rats with at least one tumor) was evaluated in terms of total mammary tumors per group (data not shown) and histologically confirmed mammary ACs only (Table 1). The majority of NMU-induced tumors were histologically AC. The distribution of total non-AC (fibroadenomas) per group (including palpable and nonpalpable tumors) was as follows: control, 4; TAM 0.1 mg, 1; TAM 1.0 mg, 0; TAM 3.0 mg, 4; TAM 10 mg, 2; LAS 0.1 mg, 6; LAS 1.0 mg, 0; LAS 3.0 mg, 1; and LAS 10 mg, 0. Total and

Table 1 *Prevention of NMU-induced mammary carcinoma*

Treatment	Adenocarcinoma incidence[a] % (rats with tumors/rats at risk)	Adenocarcinoma multiplicity[b] mean (SD)	Mean tumor volume[c] cm^3 (SD)	Tumor latency mean days (SD)
Vehicle (control)	75% (15 of 20)	3.1 (2.5)	17 (17)	101 (25)
TAM 0.1 mg/kg	60% (12 of 20)	1.4[d] (1.5)	7.2 (18)[e]	116 (26)
TAM 1 mg/kg	60% (12 of 20)	1.2[f] (1.3)	1.9 (5)[f]	117 (24)
TAM 3 mg/kg	10%[e] (2 of 20)	0.2[g] (0.6)	1.2 (3.3)[g]	131 (6)[h]
TAM 10 mg/kg	15%[e] (3 of 20)	0.2[g] (0.5)	1.2 (4.0)[g]	130 (11)[h]
LAS 0.1 mg/kg	65% (13 of 20)	1.7[d] (1.9)	9.3 (19)	120 (18)
LAS 1 mg/kg	75% (15 of 20)	1.6[d] (1.4)	5.2 (8.1)[e]	118 (24)
LAS 3 mg/kg	65% (13 of 20)	0.9[g] (1.0)	2.0 (3.4)[f]	120 (19)
LAS 10 mg/kg	30%[d] (6 of 20)	0.4[g] (0.8)	0.55 (1.4)[g]	133 (2)[h]

[a] Percentage of rats in the group with at least one adenocarcinoma. Only histologically confirmed adenocarcinomas were included. The χ^2 test was used for statistical analysis.

[b] Total number of adenocarcinomas divided by number of rats at risk. All data were derived from the results at necropsy 18 weeks after the administration of NMU. ANOVA followed by Dunnett's Multiple Comparison Test (30) was used for statistical analysis. A linear dose-response relationship is observed by regression analysis for both TAM and LAS at $P < 0.0001$.

[c] Tumor volume was calculated from all rats at risk. A linear dose-response relationship was present for both LAS and TAM ($P < 0.01$).

[d] $P < 0.01$ compared with control.

[e] $P < 0.05$ compared with control.

[f] $P < 0.001$ compared with control.

[g] $P < 0.0001$ compared with control.

[h] $P < 0.0004$ by log-rank test adjusted for multiple comparisons.

All comparisons to control adjusted for multiple comparisons.

AC tumor incidence was decreased significantly only at the two highest doses (3 mg/kg and 10 mg/kg) of TAM and at the highest dose (10 mg/kg) of LAS. TAM at 3.0 mg/kg was the most effective treatment, reducing AC incidence from 75% in the controls to 10%. There was no obvious stepwise decline in tumor incidence with respect to LAS or TAM dose, although the two higher doses of TAM were more effective than the two lower doses. Similarly, the 10 mg/kg dose of LAS had a greater reduction of tumor incidence than any of the lower doses of LAS. The incidence data (Table 1) includes palpable and nonpalpable AC. Nonpalpable ACs were observed at necropsy and were, in general, more prevalent in the treated than the control groups. The number of animals exhibiting only nonpalpable AC at termination as a function of treatment was as follows: control, 0; TAM 0.1 mg, 2; TAM 1.0 mg, 7; TAM 3 mg, 0; TAM 10 mg, 0; LAS 0.1 mg, 2; LAS 1.0 mg, 1; LAS 3 mg, 4; and LAS 10 mg, 1.

Tumor multiplicity was evaluated as both total mammary tumors and for histologically confirmed AC only. In addition, multiplicity was assessed in two ways: (*a*) total tumors/total rats at risk or (*b*) total tumors/tumor-bearing rats. When assessed in terms of total tumors/total rats at risk, tumor multiplicity was significantly reduced in both TAM- and LAS-treated groups, and this reduction was linearly related to dose (Table 1). LAS reduced tumor multiplicity 65% and 89% at doses of 3 mg/kg and 10 mg/kg, respectively. The greatest degree of reduction of multiplicity was in the high-dose TAM group in which multiplicity was reduced from a mean control level of 3 tumors/rat to 0.20 tumors/rat (Table 1). When assessed in terms of the mean number of tumors/total tumor-bearing rats, a similar result was found. Thus, considering only tumor-bearing rats, the control rats had a multiplicity of 4.1 ± 2.0 tumors/rat, whereas the 3 mg/kg and 10 mg/kg LAS groups had 1.4 ± 0.9 and 1.3 ± 0.8 tumors/rat, respectively.

Both TAM and LAS reduced the total tumor number/group in a dose-responsive manner, whether assessed in terms of total mammary tumors (data not shown) or AC only (Fig. 2). There was a striking >85% reduction in total tumors in the presence of the highest dose of LAS or TAM. This result is reflective of the multiplicity data (Table 1) and illustrates the profound effect these agents have as chemopreventive agents when assessed in terms of total tumor burden.

Tumor volume was also markedly suppressed by treatment with SERMs and this suppression was dose related whether assessed in terms of total animals at risk (Table 1) or tumor-bearing rats only. Using the former, tumor volume was decreased by 70% and 90% at the highest dose (10 mg/kg) of TAM and LAS, respectively. Considering only the tumor-bearing rats from each group, the total tumor volume was 20 ± 16 cm^3/rat for the controls *versus* 3.9 ± 3.9 and 2.2 ± 2.2 cm^3/rat for the 3 mg/kg and 10 mg/kg LAS groups, respectively. The distribution of histologically confirmed AC that were nonpalpable (*i.e.*, could not be accurately measured) among the test groups was as follows: control, 0; TAM 0.1 mg, 2; TAM 1.0 mg, 11; TAM 3 mg, 0; TAM 10 mg, 0; LAS 0.1 mg, 3; LAS 1.0 mg, 1; LAS 3 mg, 5; and LAS 10 mg, 1. These tumors were assigned volumes of zero in calculations.

Effect of Treatment on Weight Gain. All groups of rats in the prevention study continued to gain weight over the course of the study (Fig. 3, *A* and *B*). However, there was an evident suppression of weight gain in all of the treated groups beginning at week 8 in the LAS-treated group and week 4 in the TAM treated-group; by week 18, all of the TAM-treated groups were significantly lower than the controls, the difference for the two highest doses being highly significant ($P < 0.0001$). In LAS-treated groups, a similar effect was seen, but by week 18 only the two lowest doses of LAS, but not the two

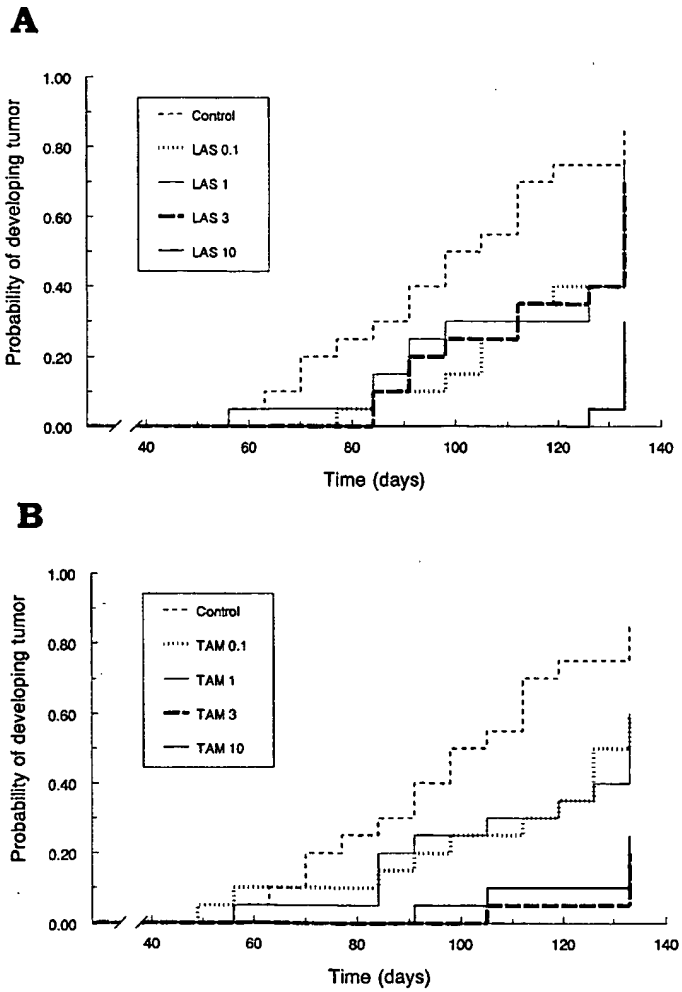

Fig. 1. Kaplan-Meier Life Table curves for cumulative mammary tumor incidence in the controls *versus* LAS- (*A*) and TAM-treated (*B*) rats. Ordinate, the proportion of total animals surviving per unit time without a tumor (1.0 represents 100% tumor-free animals). Abscissa, days post-NMU; LAS 10 mg/kg, $P < 0.0004$; TAM 3 mg/kg and 10 mg/kg, $P < 0.0004$ by log-rank test; and all other pairwise comparisons, nonsignificant.

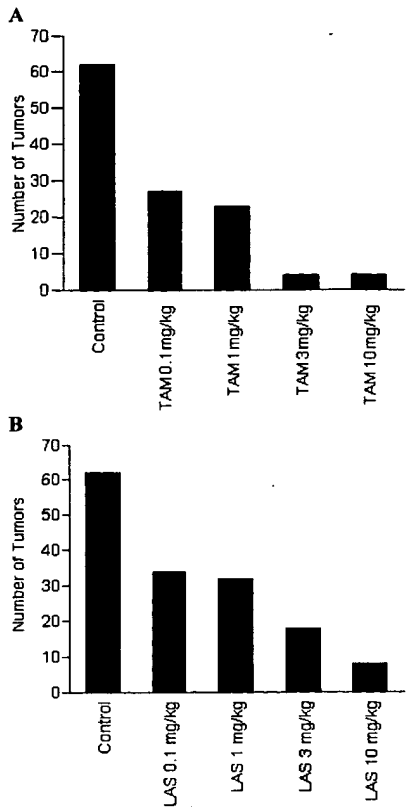

Fig. 2. Total number of mammary adenocarcinomas by treatment group. The values shown are the total number of histologically confirmed adenocarcinomas in each group at the conclusion of the chemoprevention study as described for Table 1. *A*, TAM compared with the controls. TAM at 0.1 mg/kg was significantly different from the controls with $P < 0.001$, and all other doses of TAM were significantly greater than the control at $P < 0.0001$ by using the χ^2 test. *B*, LAS compared with the controls. LAS at 0.1 mg/kg was significantly different from the controls at $P < 0.05$, LAS at 1 mg/kg at $P < 0.01$, and LAS at 3 mg/kg and 10 mg/kg at $P < 0.0001$ as assessed by the χ^2 test. For both LAS and TAM, the dose response effect was significant by linear regression analysis at $P < 0.001$.

higher doses, were significantly lower than the controls. When averaged over time, TAM but not LAS produced significantly reduced weight gain relative to the controls, as assessed by ANOVA for repeated measures.

LAS Plasma Levels. The plasma concentrations of LAS in the rats of the prevention study were roughly proportional to dose throughout the dosing range of 0.1–10 mg/kg when examined at 2 h after oral dosing (Table 2). This time corresponded to the T_{max} for oral dosing, and the $t_{1/2}$ was ~4 h.[4] Thus, a statistically significant reduction of tumor multiplicity was associated with plasma levels as low as 1.6 ng/ml (0.1 mg/kg dose), and a reduction of tumor volume was seen at 22 ng/ml (1 mg/kg dose).

Treatment of Established Tumors. LAS delayed the development of tumors and also had activity against established (palpable) mammary tumors. Treatment with LAS inhibited the growth of mammary tumors and produced complete regression in some tumors (Tables 3 and 4). In agreement with the previous reports of this model (14, 27), the growth rate of individual NMU-induced tumors is highly variable. Thus, some tumors rapidly progress to a large size whereas others progress slowly or even remain stable. However, the effect of LAS treatment is clear. In the control (vehicle-treated) rats, 81% of tumors increased in size by >2-fold over the test period, and 19% were stable. In contrast, among animals that were administered 10 mg/kg LAS, only 30% progressed, whereas 30% were stable and 40% regressed. One tumor regressed completely on this treatment schedule, and one additional tumor regressed completely in both the LAS 1 mg/kg and 3 mg/kg groups. The lower doses of LAS also resulted in fewer progressive tumors and produced partial tumor regressions (Table 3). The major benefit of LAS appeared to be seen at a dose of 1 mg/kg, with higher doses (10 or 100 mg/kg) showing only small additional increases in efficacy (Tables 3 and 4).

Furthermore, as expected on the basis of the results in the prevention study, in the treatment study the appearance of new second and third tumors was common in the controls and rare in the highest-dose LAS- or TAM-treated groups. For example, during the course of

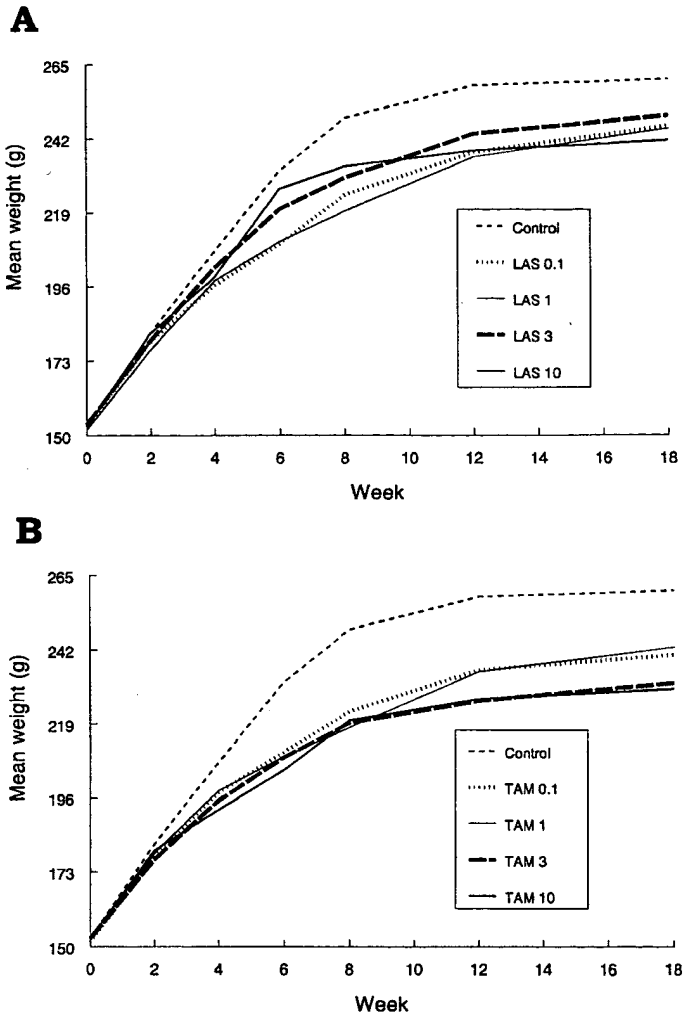

Fig. 3. The mean cumulative weight gain over time as a function of treatment group. *A*, LAS; *B*, TAM. LAS 0.1 mg/kg and 1 mg/kg less than the controls ($P < 0.01$); TAM 0.1 mg/kg ($P < 0.001$), 1 mg/kg ($P < 0.05$), and 3 mg/kg and 10 mg/kg ($P < 0.0001$) less than the controls by ANOVA for repeated measures, adjusted for multiple comparisons. All other pairwise comparisons, NS.

Table 2 *Plasma concentrations of LAS in treated rats*

Plasma samples were collected from the blood of six rats in each LAS-treated group of the prevention study of Table 1 at 2 h after the indicated oral dose on day 40 of the study. LAS concentrations were determined as described in "Materials and Methods."[a]

LAS dose (mg/kg body weight)	Mean plasma concentration LAS (ng/m) ± SD
0.1	1.3 ± 0.5
1	22 ± 5.6
3	55 ± 31
10	308 ± 64

The plasma concentrations were dose-dependent at $P < 0.0001$ by regression analysis.

[4] L.Yu *et al.*, unpublished pharmacokinetic study.

Table 3 *Chemotherapeutic effects of LAS on NMU-induced tumors*

Group	Dose (mg/kg)	Number of rats	Total no. of tumors	Progressive[a]		Stable		Partial regression		Complete regression	
				No. of tumors	%	No. of tumors	%	No. of tumors	%	No. of tumors	%
Control	Vehicle	8	16	13	81	3	19	0	0	0	0
TAM	10.0	8	8	3	38	3	38	2	25	0	0
LAS	0.1	8	11	4	36	6	55	1	9	0	0
LAS	1.0	7	11	3	27	4	36	3	27	1	9
LAS	3.0	7	11	2	18	4	36	4	36	1	9
LAS	10.0	10	10	3	30	3	30	3	30	1	10

[a] The criteria for the categories are as follows: progressive, >100% increase in size; partial regression, tumor decreased in volume by ≤50%; complete regression, tumor disappeared completely. All other tumors were placed in the stable category. The distribution of the LAS 3 mg/kg and 10 mg/kg groups were significantly different from the controls by the χ^2 test ($P < 0.05$).

treatment of the eight vehicle-treated rats of the experiment in Table 3, eight new tumors appeared. However, in the 10 rats of the 10 mg/kg LAS-treated group, no additional tumors appeared. Thus, in animals bearing one tumor the administration of LAS at 10 mg/kg totally suppressed the appearance of secondary and tertiary tumors.

The majority of NMU-induced tumors grew progressively in the control rats (Table 3). Although it is useful to consider the tumors individually in view of the variable growth rates, as mentioned in the foregoing discussion, an assessment of the group mean and median percentage change from baseline to termination for the tumors in rats treated with TAM and LAS also provides useful information (Table 4). The mean volume of the control tumors increased 330% from appearance to week 19 (or termination if necessary for very large tumors). By contrast, the mean or median increase in tumor size in all groups treated with LAS or TAM was much smaller. The mean increase for LAS at 3 mg/kg and 10 mg/kg was only 27% and 52%, respectively, and this increase was found to be statistically less than in the controls.

DISCUSSION

Although several antiestrogens have been evaluated in the NMU-induced mammary carcinoma model, differences in dose, route of administration, and methods of evaluation make conclusive comparisons across studies difficult. Furthermore, for such comparisons to be meaningful for clinical consideration, issues of exposure and toleration may be paramount. However, with these limitations in mind, it is of interest to compare the results here with those of previously tested antiestrogens in this model.

A study of raloxifene treatment of 7,12-dimethylbenz(*a*)antracene-induced rat mammary carcinomas indicated that raloxifene produced a substantial inhibition of tumor growth but was inferior to equivalent doses of TAM (28). More recently, the chemopreventive activity of TAM has been studied in several dosing schedules in the NMU-induced tumor model and compared with the activity of raloxifene in the same study (26). Both TAM and raloxifene, when dosed for 8 weeks on a daily basis (100 μg/rat) starting 2 weeks after NMU exposure, greatly delayed the appearance of tumors, although TAM was more potent and effective. Raloxifene was also effective in delaying the emergence of tumors when dosing was delayed to 7 weeks after NMU exposure, but TAM was more effective. In this study, we did not examine the effect of delayed treatment after NMU exposure, but LAS suppressed the emergence of second tumors when dosing was delayed until after emergence of the first tumor ("treatment protocol").

Table 4 *Inhibitory effect of SERMS on tumor growth (treatment model)*

Tumor volumes are shown as percentages (mean and median values) relative to the values at the onset of LAS or TAM exposure.

Group	No. of tumors	Tumor volume: Percentage of change from baseline[a]	
		Mean ± SD	Median
Vehicle	16	330 ± 297	237
TAM 10 mg/kg	8	203 ± 419	−16
LAS 0.1 mg/kg	11	86 ± 147	14
LAS 1 mg/kg	11	113 ± 271	−15
LAS 3 mg/kg	11	27 ± 130[b]	−11[c]
LAS 10 mg/kg	10	52 ± 175[b]	51[c]

[a] The dose response across LAS doses and controls: $P < 0.056$ by linear regression analysis.

[b] $P < 0.05$ *vs.* control by ANOVA followed by Dunnett's test (25).

[c] $P < 0.05$ *vs.* control by median test adjusted for multiple comparisons.

The SERMs droloxifene and pyrrolidino-4-iodotamoxifen have been evaluated for the treatment of NMU-induced carcinomas. Droloxifene at 6 mg/kg daily induced regressions of 22% of tumors and rendered 22% of the tumors stable, whereas TAM produced 28% regressions in that study (28). Pyrrolidino-4-iodotamoxifen at a dose of 1 mg/kg produced 58% regressions (a >50% decrease in tumor size) of NMU-induced tumors, whereas TAM produced 42% regressions at the same dose in this study (29). By comparison, in our studies, the highest dose of LAS tested (10 mg/kg) produced 40% regressions, and TAM produced 25% regressions. All of these agents, therefore, are active in this model, and direct comparative studies would be necessary to determine whether one has superior activity.

Anzano and colleagues (30) have suggested that combination chemopreventive therapy could provide better protection than the antiestrogen alone. They showed that raloxifene combined with 9-*cis*-retinoic acid provided a greater delay of tumor emergence and reduced tumor burden in the NMU-induced mammary tumor model more than raloxifene alone. Our study provides a basis for exploring similar combinations of LAS and other chemopreventive agents.

TAM, and to a lesser extent, LAS, administration over an 8-week period resulted in depressed weight gain compared with the untreated controls. Although food consumption was not measured in this study, it is unlikely that SERM administration resulted in appetite suppression because weight gains remained suppressed during the 10-week period during which animals received no SERMs (Fig. 3, *A* and *B*). However, *in vivo* and *in vitro* studies in animals, and studies in humans, have shown that TAM inhibits GH secretion by the pituitary gland, and that TAM treatment significantly reduced GH response to the growth hormone releasing factor and reduced plasma insulin-like growth factor I levels (31, 32). There is also evidence that TAM can act peripherally by blocking tyrosine phosphorylation of the insulin-like growth factor I-IR β subunit (33). Hence, one likely explanation for the weight gain suppression observed in this present study is that TAM and LAS exert secondary effects at the level of the hypothalamus pituitary axis by suppressing GH secretion.

In summary, LAS is active in both the chemoprevention of NMU-induced mammary carcinomas and in the treatment of established mammary carcinomas in this model, with no apparent toxic side effects. Its efficacy in this model was similar to that of the established antiestrogen TAM. Clinical studies will be necessary to establish

whether this agent will reduce breast cancer incidence in women receiving this agent for the treatment of osteoporosis.

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